Developmental signaling: **Notch signals Kuz it's cleaved**

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Recent experiments with Kuzbanian, a disintegrin metalloprotease that is required during development for lateral inhibitory signaling, suggest that signaling molecules of the Notch family may guide cell fate only after they are activated by proteolysis, and that the proteolysis may be catalyzed by Kuzbanian.

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Understanding of the molecular details of how the transmembrane signaling protein Notch transduces signals from adjacent cells has taken several giant steps forward in recent months with the discovery of the *kuzbanian* (*kuz*) gene and the elaboration of the pathway that regulates the cleavage and intracellular traffic of Notch protein. Notch signaling is responsible for a wide variety of developmental processes, most notably lateral inhibition (for recent reviews, see [1,2]).

Lateral inhibitory signals are emerging as a basic element of cellular differentiation in numerous structures, including the nervous system, muscle, and hematopoietic cells. In contrast to an inductive signal, Notch-mediated lateral inhibitory signals restrict a cell's destiny, preventing its differentiation into a particular cell fate when it is in direct contact with another cell that bears a ligand for Notch. The level of expression of the Notch family member, the strength of its signal and the quantity of ligand on a neighboring cell appear to be closely balanced by a feedback mechanism that is designed to apportion subsets of cells from equivalent groups into distinct fates [3,4]. The ligands which are thought to trigger Notch signaling are a family of molecules that contain a DSL domain (for Delta–Serrate–lag-2; see [5] for review). The Notch signaling cascade has numerous components; a growing number of proteins adhere to and, presumably, regulate Notch (Figure 1; see [1] for review). Moreover, other important developmental signaling pathways — most notably the Wnt signaling pathway — intersect with the Notch pathway [6]. Like many signal transduction pathways, Notch signaling ultimately results in regulation of gene expression, which in turn alters cellular fates during development.

The importance of Notch signals is also becoming evident in a wide variety of processes beyond developmental biology. Truncated Notch proteins cause lymphoid [7] and breast [8] neoplasia making Notch of central interest to oncologists and cancer researchers. For the neuroscientist and neurologist, mutations in Notch3 are the etiology of an adult-onset neurodegenerative disorder, CADASIL [9]. Finally, recent studies have tied together Notch family members and the presenilins, genes underlying some cases of familial Alzheimer's disease [10]. Investigators from a wide range of fields are therefore watching the developments in Notch biology closely.

Notch activation

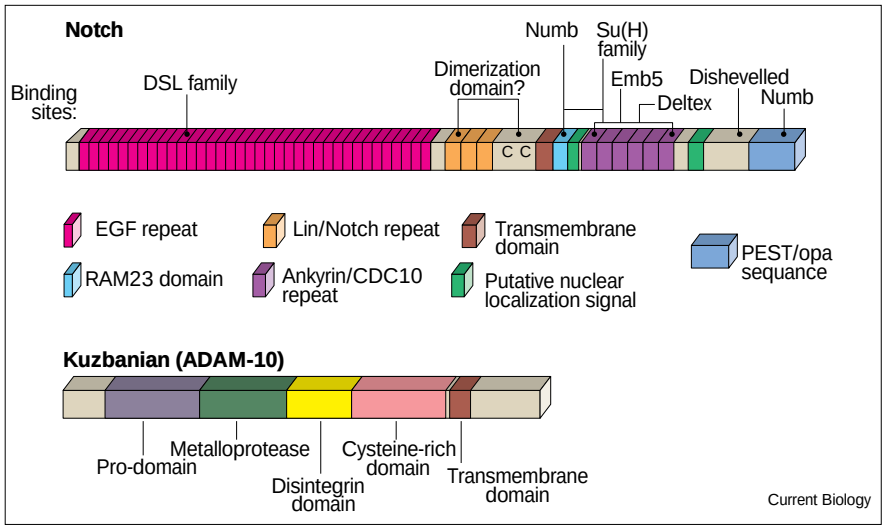
The biochemical details of Notch activation by its ligand have remained somewhat elusive. The expression of DSL-containing ligands on the surface of cells enables them to adhere to Notch-expressing cells. Beyond that, the mechanism of transmission of the intracellular signal is unknown, but there are a number of intriguing clues. Deletion experiments have suggested that the extracellular Lin–Notch repeats (LNR) and a pair of extracellular cysteines near the single transmembrane domain of Notch (see Figure 1) are involved in regulating activation. Moreover, Notch proteins may self-associate, perhaps via intermolecular disulfide bridges from the conserved cysteines.

Even before the identification of *kuz*, extracellular proteolysis was thought to occur given that, for each of the Notch family members examined, the majority of the endogenous protein is found in a cleaved form. Recent characterization [11] suggests that full-length Notch protein never reaches the cell membrane. Instead, a truncated protein of 100–120 kDa (termed Notch^{TM}), including the intracellular domain and a small segment of extracellular protein, may be the predominant form of Notch on the plasmalemma. In fact, this fragment of Notch protein, which probably lacks the EGF-like (epidermal growth factor-like) repeats, is found in a protein complex that adheres to the ligand Delta, possibly via an interaction with the cleaved remainder of the extracellular domain, Notch^{EC}.

To understand the role of proteolysis fully, it is important to know the structure and activity of the cleaved fragments. Work on the proteolysis of the *Caenorhabditis* Notch homologue GLP-1 indicates a site of cleavage between the LNR domain and the transmembrane domain [12]. In mouse Notch-1, a similar cleavage occurs at a site on the extracellular domain (residue 1655), generating fragments that are inactive in an assay for Notch activity that involves inhibition of muscle-specific promoters [13]. Cleavage of the full-length Notch at such a site, in the various species studied, is likely to generate proteolytic fragments of approximately the same size as the Notch^{TM} and Notch^{EC}

Figure 1

(a) The general structure of a Notch family member, and some of the proteins that adhere to Notch. (b) The structure of Kuzbanian (ADAM-10).



fragments; these proteins are probably inactive until contacted by a ligand.

Inside the cell, Notch signal transduction relies upon proteins of the Suppressor of Hairless or Su(H) family of transcription factors, which are somehow energized by their interaction with Notch. Su(H) proteins adhere to the intracellular RAM23 [14] domain of Notch (Figure 1) but also require the ankyrin/CDC10 repeats of Notch for their subsequent activation [15]. It is widely agreed that the activities of Notch are mediated for the most part by the Su(H) family and its effects on gene transcription (for an exception, however, see [16]). However, whether the Notch intracellular domain acts in the cytoplasm to trigger Su(H) activation or whether it travels to the nucleus to do so [17] remains controversial. Intracellular Notch fragments are found in the nucleus only after truncated forms of Notch have been expressed. Some experiments reveal an intracellular cleavage that leads to the release of a small quantity of a cytoplasmic Notch polypeptide that travels to the nucleus [13], but it has not been determined whether this cleavage occurs following ligand activation or if it is required for signal transduction.

Kuzbanian's role in cell fate decisions

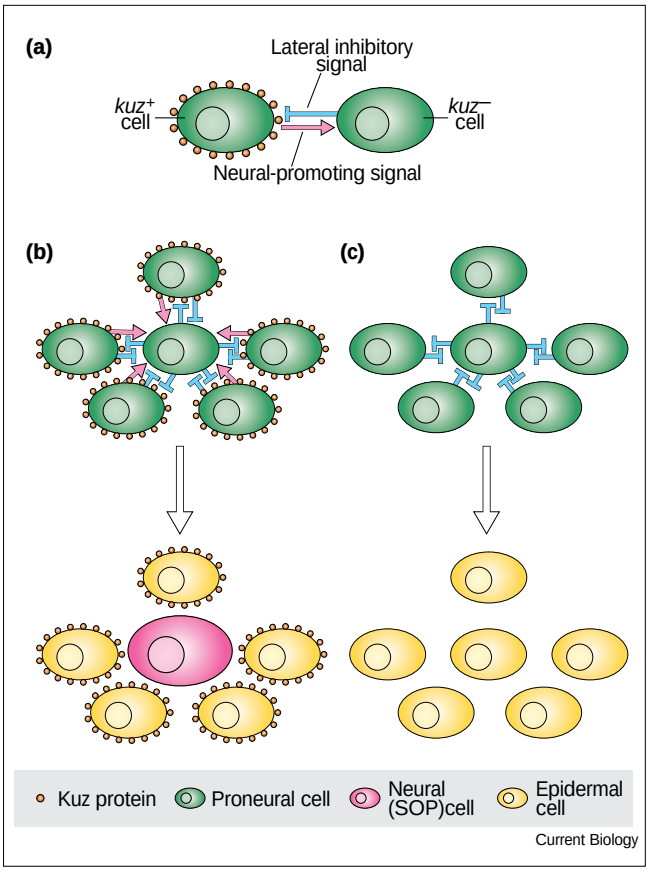
The newly identified Kuz molecule, named after a Muppet character whose exaggerated tufts of hair resemble the excess bristle formation in patches of *kuz*-deficient cells, was discovered because of its role in producing a lateral inhibitory signal during development of the sensory bristles of adult fruitflies [18]. But *kuz* is interesting and unique because it appears to drive changes in all the cells participating in lateral inhibition (Figure 2), rather than only in the recipient of the signal. Not only is *kuz* required for the lateral inhibitory signal in the receiving cell, but it also appears to be important for specification of the parent cell of the sensory bristle apparatus, the sensory organ precursor (SOP). The evidence of a reverse signal that is required for development of the SOP contradicts the model in which all cells in a proneural cluster are destined to give rise to an SOP unless restricted by the lateral inhibition machinery that uses Notch; this layer of complexity needs further illumination. In addition, *kuz* also plays a role in the fidelity of axonal tracking that occurs during the development of neurons of the central nervous system, a function that has not previously been clearly associated with Notch signaling [19].

Protease function is required

Recent studies reveal how Kuz may be involved in producing the lateral inhibitory signal [20]. The fly, mouse and *Xenopus* versions of Kuz are members of a growing family of membrane-associated metalloproteases that have a disintegrin domain and a cysteine-rich domain, collectively called the ADAM family (for a disintegrin and metalloprotease domain) [21]. The name conjures up images of the Garden of Eden, a fitting reference for the founding members of the family, sperm proteins called fertilins which play a role in sperm–egg fusion, and the snake venom metalloproteases which cause hemorrhage. Kuz is an ortholog of ADAM-10 (MADM), which has proteolytic activity against numerous substrates including myelin basic protein, although its own natural substrate is unknown [22,23].

Although the Kuz protein has numerous potential functions encoded within its extracellular domain, its proteolytic activity was shown to be essential for endowing cells with the ability to respond to a lateral inhibitory signal. Dominant-negative forms of Kuz were created by deleting the metalloprotease domain or by mutating a glutamic acid residue in the conserved catalytic site sequence

Figure 2



Kuzbanian is required for both responding to the lateral inhibitory signal and transmitting a neural-promoting signal in specification of the sensory organ precursor (SOP). **(a)** The dual function of Kuz. **(b)** Mosaic analysis showing a *kuz*$^{-}$ cell surrounded by wild-type cells. The *kuz*$^{-}$ cell gives rise to the SOP (neuroblast), showing that Kuz is required in the receiving cell (cell autonomously) for lateral inhibition. Kuz is also upstream of Notch in lateral inhibitory signaling. **(c)** In a cluster of *kuz*$^{-}$ cells, lateral inhibitory signaling occurs but no neuroblasts emerge because of the lack of a neural-promoting signal transmitted by Kuz (non-autonomously).

HEXGHNXGXXHD (using the single-letter amino-acid code, with X as any amino acid) [20]. The dominant-negative mutant proteins inhibit the endogenous Kuz either by inducing the formation of heterodimers of mutant and wild-type Kuz — by analogy to the dimerization of other ADAM family members — or by competing for substrate proteins.

Using a murine version of the dominant-negative Kuz (mKuz^{DN}), Pan and Rubin [20] were able to show that Kuz proteins have a conserved function in vertebrates. In *Xenopus*, formation of the primary neurons, the first neurons to emerge from the neural tube, is regulated by lateral inhibitory signals through XNotch and its ligand XDelta-1 [24]. Addition of mKuz^{DN} causes an increase in the number of cells that differentiate into primary neurons, in a phenotype strikingly similar to that seen with a dominant-negative XDelta-1, confirming that Kuz is necessary for a lateral inhibitory signal in vertebrates.

How does Kuz direct both lateral inhibition and SOP specification signals from adjacent cells? The answer is not yet known. Several members of the ADAM family release fragments of their extracellular domain upon maturation. A released fragment may then travel to adjacent cells to exert its function. It is also possible that a fragment of a protein cleaved by Kuz might act as local inducer of differentiation.

Kuz cleaves Notch

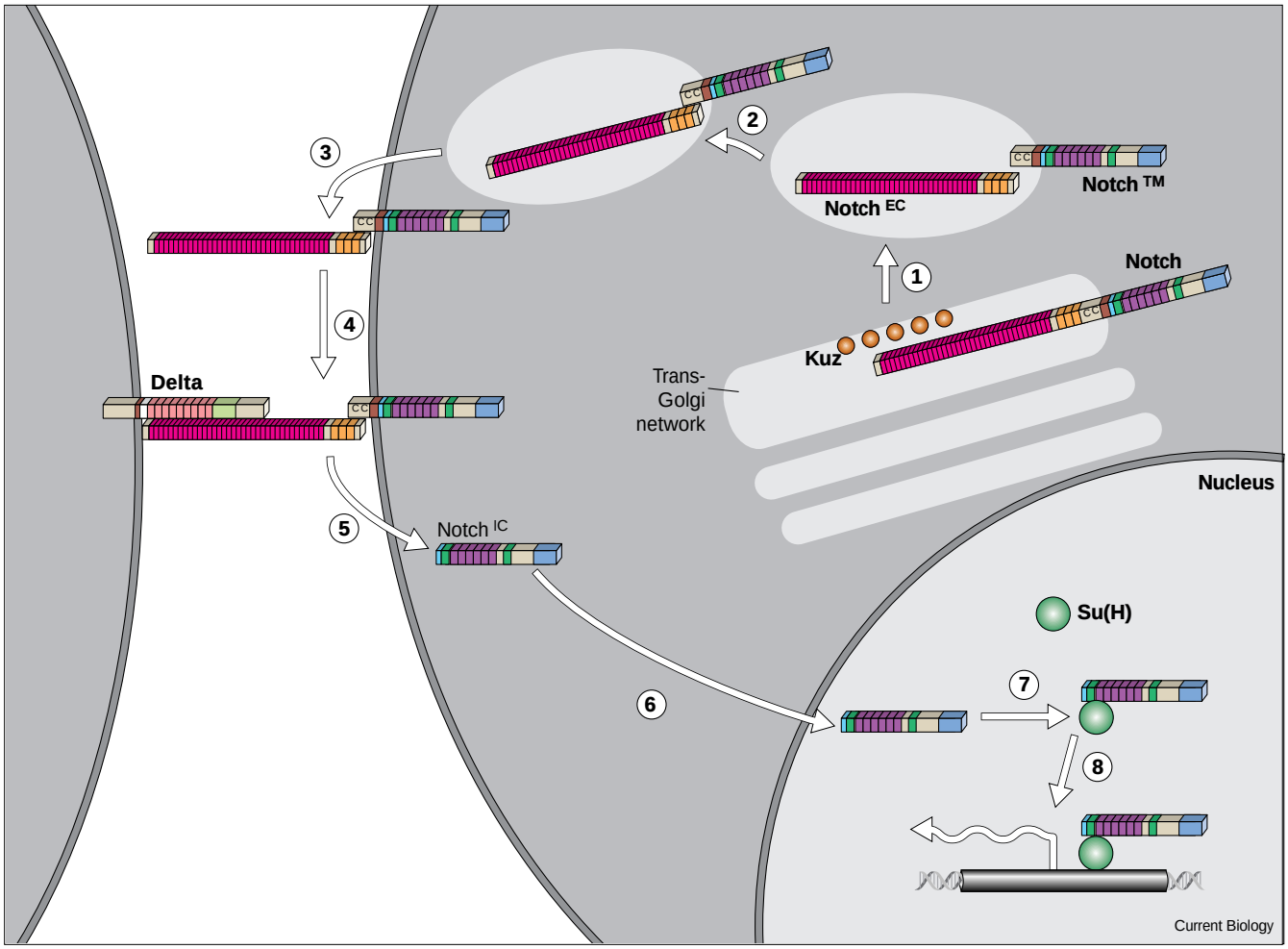
Notch, a lateral inhibitory cleaved protein in search of a protease, does indeed appear to meet Kuz, a lateral inhibitory protease in search of a substrate. The *kuz* gene interacts in a dosage-sensitive manner with the *Notch* gene, and operates genetically upstream of *Notch*, making an intimate interaction likely [20]. In *Drosophila* cells expressing Notch, a 100 kDa proteolytic fragment of Notch — presumably Notch^{TM} — is no longer found in the cells when the dominant-negative Kuz^{DN} is introduced. The cleaved Notch fragment is also lost from *kuz*-deficient embryos and is reduced when Kuz^{DN} is expressed from a heat-shock promoter. These experiments show that Kuz activity is required for the processed form of Notch to appear. If the processed form of Notch is the only form that binds ligand, then cleavage of Notch by Kuz would serve as an essential preliminary step towards the ultimate activation of Notch by a ligand, and would also explain the requirement for Kuz when cells are responding to a lateral inhibitory signal (see below). Other roles for Kuz in lateral signaling are not excluded by these experiments. For example, Kuz^{DN} appears to reduce the quantity of uncleaved Notch polypeptide [20], in addition to eliminating *Notch* cleavage. Thus, Kuz may also affect Notch indirectly, through effects on its synthesis, trafficking or metabolism.

Kuz certainly appears to have roles in development and differentiation other than the cleavage of Notch, as studies of its ortholog, the ADAM-10 protease [22], have implied. *Drosophila kuz*-null embryos have a more severe phenotype, with a greater number of excess neurons than *Notch*-null embryos. Moreover, a *kuz*-deficient mouse generated in the Rubin lab (D.J. Pan, personal communication) dies earlier in development than the *Notch1* knockout mouse. These observations, and the finding that Kuz is important for axonal pathfinding, are consistent with the hypothesis that the Kuz protease has substrates other than Notch that function during the development of vertebrates and invertebrates.

Model of Notch activation

What do these studies teach us about the mechanism of signal transduction from Notch? A hypothetical model is

Figure 3



Model of Notch signal transduction. (1) Full-length Notch is cleaved by Kuz at Notch's extracellular domain, in the trans-Golgi apparatus (constitutive cleavage). (2) Notch^{TM} adheres to the Notch^{EC} fragment. (3) The heterodimer is transfered to the plasma membrane. (4) Notch^{EC}, the fragment of the complex containing the EGF-like domain, adheres to a ligand such as Delta. (5) Activation of Notch^{TM} and release of the membrane tether by proteolysis (possibly ligand-dependent, possibly proteosome-mediated). (6) Migration of free Notch intracellular fragment (Notch^{IC}) to the nucleus. (7) Association with a member of the Su(H) family of transcription factors. (8) Binding of the complex to specific sites on DNA and the regulation of downstream gene transcription.

depicted in Figure 3. As Kuz activity is essential for cells to receive a lateral inhibitory signal, the cleavage of Notch may be of critical importance in generating a receptor that is capable of activation. Kuz is thus the best candidate for a constitutive protease that acts in the trans-Golgi network (step **1**). By a mechanism that is not yet fully understood but may involve two conserved extracellular cysteines, the extracellular fragment Notch^{EC} adheres to the membrane-spanning fragment Notch^{TM} (step **2**). The complex is then transfered to the plasmalemma as a mature protein that is capable of interacting with a ligand on another cell (step **3**). The ligand binds to the EGF-like repeats of the Notch^{EC} fragment, forming a ternary complex (step **4**). How the binding of ligand triggers signal transduction remains unclear. Possible mechanisms include dimerization, dissociation of Notch^{EC} from Notch^{TM}, or a second proteolysis step (step **5**), or any combination of the three. Intracellular proteolysis would release the tethered intracellular domain (Notch^{IC}) which can then translocate to the nucleus, aided by its nuclear localization signals (step **6**). Notch^{IC} associates with Su(H) proteins (step **7**) via the RAM23 domain, but whether this occurs naturally in the cytoplasm or nucleus *in vivo* is unknown. Finally, the ankyrin repeats of Notch^{IC} interact with members of the Su(H) family of transcription factors (step **8**), and so direct transcriptional regulation and the subsequent, downstream effects upon cell fate. Although many steps are still speculative and not yet fully understood, it is clear that with the arrival on stage of Kuz, our understanding of Notch signaling is no longer in its infancy.

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